ANAPHYLATOXIN AND ANAPHYLAXIS

XI. ULTRA-FILTRATION AND FRACTIONATION OF ANAPHYLATOXIN *

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SYNOPSIS

Despite the large amount of study on the nature of anaphylatoxin and of the mechanism of its formation, little if any attempt has been made to isolate the toxic principle on one or the other of the protein fractions of the serum. Various serum principles having physiologic activity, but of unknown chemical nature, have been isolated on the protein fractions of serum. The association of these active principles is often so close as to suggest that they may themselves be part of one of the serum proteins. Thus Alex. Schmidt,[1] by diluting and passing CO_2 through blood plasma precipitated a "fibrinoplastic" substance which was able to clot hydrocele fluid. Seng[2] quantitatively isolated diphtheria antitoxin on the water-soluble globulin of immune serum. Fuld and Spiro[3] found that the rennin-inhibiting activity of normal serum is associated with the water-soluble globulin, while the water-insoluble globulin on the other hand has a distinct milk coagulating action. Pick[4] made an extensive study of the various immunity principles of serum and found antitoxins, lysins and agglutinins to be associated with one or the other globulin fractions. The work of

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* For Parts I-X, see this Journal, 1917, 20, p. 499; also, Jour. Am. Med. Assn., 1917, 68, p. 1524.

1 Arch. f. Anat., Physiol., u. wissensch. Med., 1862, p. 461.

2 Ztschr. f. Hyg. u. Infectionskrankh., 1899, 31, p. 513.

3 Ztschr. f. physiol. Chem., 1900, 31, p. 140.

4 Beitr. z. chem. Phys. u. Path., 1902, 1, p. 351.

this author and of Seng has been practically applied to the concentration of antitoxins by Gibson,[5] Banzhaf[6] and others.

While it is entirely possible that these active principles of the serum may be relatively simple substances, attached by adsorption or otherwise to the serum proteins, the other possibility exists that the proteins themselves may be physiologically active. The fact remains that up till now no clear evidence of immune bodies freed entirely from serum proteins has been presented, and such high authority as Landsteiner[7] thinks that these substances may actually be albuminoid in character.

It would be of great interest to determine whether the anaphylatoxic principle can be isolated on serum proteins. Such procedure, if possible, would not only furnish an approach to the discovery of the nature of the toxic principle, but might eventually throw light on the mechanism of its formation. We have, accordingly, applied various methods of fractionation of serum proteins to this investigation and in addition to this have attempted to locate the toxic principle by more recently devised physico-chemical methods.

Before entering into the experimental part, it would be well to outline very briefly the prevailing notions of the number and nature of the serum proteins and definitely to define the meaning of the terms "euglobulin," "pseudoglobulin," etc., frequently used in this paper.

The water-insoluble globulin of serum was first thoroughly studied by Panum.[8] He obtained this protein by simple dilution, or by dilution with subsequent passage of CO_2 or addition of acetic acid. This author gave to the water-insoluble globulin the name "serum-casein." Kühne[9] failed to find casein characteristics in this substance and gave it the name "paraglobulin." Heynsius[10] found that this paraglobulin, besides being obtained by the methods just mentioned, could be separated by the saturation of serum with NaCl. Aronstein[11] was the first to show that the paraglobulin could be flocked out by dialysis of serum against distilled water. These dialysis studies were confirmed and extended by Alex. Schmidt.[12]

[5] Jour. Biol. Chem., 1906, 1, p. 161.

[6] Med. Rec., New York, 1909, 75, p. 581; Collected Studies from the Research Laboratory, Department of Health, City of N. Y., 1911, p. 153.

[7] Kolle u. Wassermann Handb. d. path. Mikro-org., 1913, 2, p. 1241.

[8] Virchow's Arch. f. path. Anat., 1852, 4, p. 23.

[9] Kühne's Lehrbuch der physiol. Chemie, 1868, p. 168, vited from Hammarsten, Erg. d. Physiol., 1, part 1, p. 333.

[10] Pflüger's Arch. f. Physiol., 1869, 2, p. 1.

[11] Pflüger's Arch. f. Physiol., 1874, 8, p. 75.

[12] Beitr. d. Anat. u. Physiol. als Festgabe Ludwig gewidmet., 1874.

Hammarsten[13] was led to believe that none of the methods alluded to completely separated the paraglobulin from serum, but that such separation could be achieved by a method long ago described by Denis.[14] This procedure consisted simply in saturating serum with $MgSO_4$. Hammarsten thought that all the globulin precipitated by this method was of the same nature. This idea was disputed by Burckhardt,[15] who claimed that the protein thrown out by saturation with $MgSO_4$ was not one substance, but could be separated into water soluble and insoluble portions. Hammarsten objected to this on various grounds, but many other workers brought confirmation to the findings of Burckhardt.

Kauder,[16] making use of the discovery of Méhu that $(NH_4)_2SO_4$ would precipitate substances of albuminoid nature, studied the fractional precipitation of serum proteins by this reagent. This investigator found that while it required full saturation with $MgSO_4$ to precipitate serum globulin, $(NH_4)_2SO_4$ had only to be added to half-saturation to accomplish the same result. In 1900, Fuld and Spiro,[3] during a study of the rennin-like and rennin-inhibiting substance of serum, made an extensive investigation of the $(NH_4)_2SO_4$ fractionation of serum proteins. They divided the total globulin secured by half-saturation with this reagent into two bodies; one, which they designated "euglobulin" precipitating between 28-33% saturation and water-insoluble, and the other, flocking out between 34-46%, water-soluble, which they named "pseudoglobulin." This terminology has been widely applied to the serum proteins. The majority of workers refer to three serum proteins—euglobulin, pseudoglobulin and albumin..

Two objections have been made to this classification. One, by Freund and Joachim,[17] who claim that there are in reality no fewer than six globulin bodies, and that these can be separated by $(NH_4)_2SO_4$ fractionation. These authors state the various globulins differ in certain physical constants, particularly in heat coagulation temperature. But it has been shown by Haslam[18] that the salting out of proteins from a mixture must be carried out in the same way as the separation of mixtures of fluid substances by fractional distillation, and that it frequently requires as many as 17 reprecipitations to obtain

[13] Pflüger's Arch. f. Physiol., 1878, 17, p. 413.
[14] Mémoire sur le Sang, 1859.
[15] Arch. f. exper. Path. u. Pharmakol., 1883, 16, p. 322.
[16] Arch. f. exper. Path. u. Pharmakol., 1886, 20, p. 415.
[17] Ztschr. f. physiol. Chem., 1902, 36, p. 407.
[18] Biochem. Jour., 1913, 7, p. 492.

pure products of the protein fractions. The numerous globulins of Freund and Joachim, then, are in reality due to incomplete separation of mixtures of the protein fractions. The marked differences in heat-coagulation temperature are not significant in their work because they did not rigidly control the acidity, salt concentration, and other factors which the work of Chick and Martin and others has shown to be highly important.

The second objection to the classification of Fuld and Spiro is made by some authors who think that there is in reality only one serum globulin. Space does not permit us to go into these objections at length, but the important work of Chick[19] merits mention in this connection. This investigator believes that only two proteins, globulin and albumin, exist in the serum. The apparent difference in water solubility of parts of the globulin is due to the formation of an adsorption complex between a part of the globulin and lecithin. This complex is water-insoluble and has the properties of euglobulin. Chick was able by addition of weak lecithin emulsions to globulin containing no phosphorus to obtain a product having in general the characteristics of euglobulin. What is more, by long continued dialysis at a slightly raised temperature, Chick was able to change all the globulin of serum to a water-insoluble condition. This is due, in her opinion, to a gradual denaturation of the water-soluble globulin with the simultaneous formation of the lipoid complex. The finding of Hardy[20] that the euglobulin contained a definite amount of phosphorus while the pseudoglobulin possessed only traces of this substance is referred by Chick to the failure to disrupt the lecithin-globulin complex. In this connection Chick points to the work of Hartley,[21] who studied the constitution of serum protein by the Van Slyke nitrous acid method, and who could find chemical differences between the whole globulin and albumin only.

It is quite possible that Chick is right in her contention and that only one globulin does exist in the serum. However, for practical purposes it is convenient to distinguish between water-insoluble and water-soluble globulin bodies. Whether euglobulin is only a complex of water-soluble globulin with lecithin or not, the fact remains that a certain quite definite amount of this substance comes down on dialysis, on dilution + acidification and on NaCl saturation, while another por-

[19] Biochem. Jour., 1914, 8, p. 404.
[20] Jour. Physiol., 1905, 33, p. 251.
[21] Biochem. Jour., 1914, 8, p. 541.

tion remains in solution. Bearing in mind that the distinction may be an unreal one, we will speak of three types of protein in the following work—the total globulin, separated from the albumin by half-saturation with $(NH_4)_2SO_4$, and in turn divided into a water soluble and insoluble portion by the procedures described in the preceding sentence.

DIALYSIS OF ANAPHYLATOXIN

The method of dialysis was first attempted, and was conducted in collodion sacs prepared by the Novy-Gorsline[22] method. These sacs were found to be greatly superior to the parchment bags ordinarily used. The first experiment was made to determine whether any part of the anaphylatoxic principle of rat serum could be isolated on the euglobulin obtained by dialysis.

The method used for the production of anaphylatoxin, whether from rat or guinea-pig serum, was the "sol-gel" agar method described by Novy and De Kruif.[23] We will describe this briefly at this point, and thereafter confine ourselves to the statement that 'the anaphylatoxin was prepared in the usual manner,' unless some deviation from this technic was resorted to.

Six rats were bled from the heart, the blood pooled and centrifugated at 8,000 revolutions. Fifteen c c of the supernatant serum were mixed with 3.75 c c of agar (0.5%) hydrosol which had been previously kept at a temperature of 37 C. for 1 hour. The mixture was shaken thoroughly, placed in cracked ice for 2 hours, and following this incubated for 30 minutes. The agar was removed by centrifugation and the resulting supernatant fluid tested for toxicity. The result is given in A of Table 1.

Four c c of the anaphylatoxin were placed in a thin collodion sac and dialyzed against running distilled water for 22 hours. The heavy precipitate of euglobulin was removed by centrifugation at 3,000 revolutions.

The supernatant fluid, B, from this centrifugation was made isotonic with 17% NaCl and found to have increased in volume to an extent that 6.15 c c of this fluid were equivalent to 4 c c of the anaphylatoxin before dialysis. The toxicity of fluid B was tested by intravenous injection into guinea-pigs. The result is given in B of Table 1.

The euglobulin precipitate, C, removed from the dialysee by the centrifugation just described, was dissolved in 1 c c of 0.85% NaCl solution. The solution, which was very opalescent, was injected and the result is recorded in C of Table 1.

The experiment just recorded makes no attempt at a quantitative estimation of the amount of toxic principle recovered, but indicates

[22] Contrib. to Med. Research, dedicated to V. C. Vaughan, 1903, p. 390.
[23] Jour. Infect. Dis., 1917, 20, p. 536.

merely that all of the active principle is not inactivated by dialysis, and moreover that it is present on both the water-soluble and insoluble fractions. An error was made in this experiment which we attempted afterward to avoid — the use of too little NaCl solution in dissolving the euglobulin precipitate. The solution obtained in this experiment was of a very low grade of dispersion, and many trials made subsequently have convinced us that a highly dispersed solution is necessary to demonstrate all of the toxic principle carried down with the euglobulin fraction.

TABLE 1

The Dialysis of Rat Anaphylatoxin

Solution	Guinea-Pig		C C Intra-venously	Serum Equiva-lent	Result
	No.	Weight			
A (Anaphylatoxin)	1	200	0.25	0.25	Nil
	2	210	0.5	0.5	+3′55″
B (Pseudoglobulin + albumin)	3	200	6.15	4.0	+4′30″*
C (Euglobulin)	4	210	1.0	4.0	+3′30″*

* The pictures of shock, the method of death, and the necropsy findings in the guinea-pigs dying from the injections of B and C correspond perfectly with that from the original anaphylatoxin A.

The euglobulin recovered from dialysis of normal rat serum was tested for toxicity as a control, and it was found that 5, 8 and even 10 c c serum equivalent of this substance produced slight if any toxic effect when injected intravenously into guinea-pigs of 200 gm. weight. Rat serum, like that of many other animal species, shows varying degrees of primary toxicity, but this toxicity has in no case been found to precipitate with the water-insoluble globulin.

Many dialyses of rat anaphylatoxin were carried out with disappointingly irregular results. Given samples of anaphylatoxin were in several instances divided into 3 or 4 equal parts, placed in collodion sacs of as nearly as possible like permeability, and dialyzed for identical lengths of time. In spite of these precautions it frequently happened that the euglobulin in one of the sacs would prove lethal to guinea-pigs while that from the others had no effect.

It appeared from any tests by dialysis that the toxic principle did not separate out exclusively on one fraction, but that a partition on the water-soluble and insoluble fractions took place. The following experiment indicates this fact clearly.

Rat anaphylatoxin prepared from rat serum by the usual sol-gel method was tested for toxicity and found to be fatal to guinea-pigs of 200 gm. weight in dose of 0.25 c c. Ten c c of this were placed in a thin collodion sac and dialyzed against running distilled water for 24 hours.

A. The precipitate was centrifugated out at 8,000 revolutions and dissolved in 10 c c of 0.85% NaCl (original volume). The solution, which was very opalescent, was cleared by the addition before injection of a few drops of N/25 Na_2CO_3. The result of the injections is given in A, Table 2.

B. After removal of the euglobulin precipitate A, CO_2 was bubbled through the supernatant and resulted in a further gummy precipitate, which was dissolved in NaCl solution as in the case of A, and cleared with alkali. The result of the injections is given in B, Table 2.

C. The supernatant remaining after the passage of the CO_2 was concentrated to 7.6 c c in a current of warm air and made isotonic with the addition of 0.4 c c of 17% NaCl solution. Result of injections is given in C, Table 2.

TABLE 2

DIALYSIS OF RAT ANAPHYLATOXIN

Solution	Guinea-Pig		C C Intra-venously	Serum Equiva-lent	Result
	No.	Weight			
A (Euglobulin, dialysis)...........	1	185	4.5	4.0	+7′30″
	2	180	3.3	3.0	+3′40″
	3	192	2.2	2.0	Very severe shock
B (Euglobulin, CO_2)..............	4	170	3.3	3.0	+5′30″
	5	195	2.2	2.0	Very severe shock
	6	190	2.7	2.5	Very severe shock
C (Pseudoglobulin + albumin)....	7	195	5.0	4.0	+4′20″
	8	190	2.5	2.0	+5′30″
	9	215	1.5	1.25	+2′30″

It will be observed that in this case a large amount of the total toxicity was recovered. The lethal dose of the anaphylatoxin employed being 0.25, the original 10 c c of serum contained 10/0.25, or 40 lethal doses; 10/3.0, or 3.3 lethal doses, were recovered from A and B, respectively, totalling 6.6 for the euglobulin, while at least 10/1.25, or 8 m. l. d. were recovered from the water soluble portion of the serum. Unfortunately, not enough of solution C remained to complete the test. Neglecting this, between 14 and 15 lethal doses were recovered from the 40 originally present.

What is the cause of the small amount of the toxic principle recovered? The collodion sacs used were very thin, and it is possible that some of the toxin might be lost by diffusion through the sac. This point was tested by dialyzing against changes of distilled water. The dialysates were pooled and concentrated in a current of warm air to a small volume, but in no case were we able to demonstrate toxicity. Experiments outlined below under "Ultra-Filtration" will

bring confirmation to the idea that very little or none of the toxic principle passes a collodion sac made in the ordinary manner. Another possibility is that the toxic principle is quite unstable, and that in the presence of distilled water it may change to a harmless product. Strength is lent to this view by the fact that euglobulin undergoes marked changes when it remains for any time in contact with distilled water. It was thought advisable, therefore, to reduce as much as possible the time consumed in dialysis, endeavoring to obtain at the same time the greatest possible yield of euglobulin.

For this purpose the method of Heynsius[24] was employed. This investigator demonstrated that when serum was exactly neutralized previous to dialysis the flocking out of euglobulin occurred with considerably greater speed than was otherwise the case, but that the contents of the sac soon became alkaline again. Consequently, to insure neutralization at the end of the reaction it was necessary to add a considerable excess of acid at the start. By this method a large amount of euglobulin can be obtained in a very short time.

Rat anaphylatoxin was prepared in the usual manner and the minimal lethal dose was found to be 0.35 c c for a guinea-pig of 200 gm. weight. Ten c c were acidified with 1.5 c c of N/5 HCl, placed at once in a thin collodion sac and dialyzed against running distilled water. A heavy precipitate had separated in 30 minutes. To insure a maximal yield the dialysis was continued for 8 hours.

A. The precipitate was centrifugated out at 8,000 revolutions and dissolved in 20 c c (twice volume), of 0.85% NaCl solution. It has been mentioned before that the toxicity of anaphylatoxic euglobulin varies directly with the degree of the dispersion of its solutions. Consequently the euglobulin solution just made was tested for toxicity in the following manner.

1. The first tests were made with the double volume 0.85% NaCl solution A.
2. After test of 1 the balance of the solution (11 c c), were diluted to 22 c c (4 times volume), and tested.
3. The balance of 2, after the test injections, was diluted to 24 c c with 0.85% NaCl (6 times volume), and tested.
4. After the injection of the tests of 3, 1 c c of N/25 Na_2CO_3 was added to the balance of the solution. The solution, which through 1, 2 and 3 had been opalescent, became perfectly clear. It was tested at once.

The result of all the tests is recorded in Table 3.

The result of the experiment recorded in Table 3 is far better than the previous ones. By dilution and addition of alkali to form the so-called "salt alkali globulin," the m. l. d. of the euglobulin recovered by this method of dialysis was reduced to between 1.0 and 1.17 c c, a yield of at least 32% of the total original toxicity.

[24] Pflüger's Arch. f. Physiol., 1876, 12, p. 549.

That practically all of the water insoluble globulin was removed by this 8-hour acid-dialysis is shown by the fact that bubbling of CO_2 through the supernatant failed to bring down any further precipitate. Similar experiments have confirmed this method as the most effective one for recovering the euglobulin toxicity by dialysis.

TABLE 3
ACID DIALYSIS (HEYNSIUS) OF ANAPHYLATOXIN

Solution	Guinea-Pig		C C Intra-venously	Serum Equiva-lent	Result
	No.	Weight			
1 2 × volume	1	180	2.0	1.0	Fair shock
	2	190	3.0	1.5	Severe shock
	3	178	4.0	2.0	+3′50″
2 4 × volume	4	225	6.0	1.5	+3′10″
3 6 × volume	5	182	6.0	1.0	Severe shock
4 6 × volume + alkali	6	195	6.0	1.0	Near-kill
	7	180	7.0	1.17	+4′50″

It has been shown by Chick[19] that by long continued dialysis at a slightly higher than ordinary temperature all of the globulin in serum can be made water insoluble. We attempted this experiment, thinking that in this way we might recover not only the toxic principle attached to the ordinarily present water-insoluble globulin, but that the portion which usually remained with the water-soluble fractions might precipitate as this changed to the insoluble form. The temperature used for this experiment was 45 C., a constant temperature being maintained by suspending the dialyzing sac in a Roux bath.

Eleven c c of an anaphylatoxin of an m. l. d. of 0.35 c c were placed in a thin collodion sac and dialyzed under the conditions just described.

A. After dialysis for 18 hours the resulting heavy precipitate was centrifuged out and dissolved in 21.5 c c of 0.85% NaCl + 0.5 c c of N/25 Na_2CO_3 (2 × vol.).

B. The supernatant from A was bubbled with CO_2 for 10 minutes, the resulting gummy precipitate centrifuged out, and similarly dissolved in NaCl and alkali (2 × vol.).

C. The supernatant from B was returned to the sac and dialyzed for an additional 17 hours and the small resulting precipitate dissolved in the same way as A and B.

D. The supernatant from C was bubbled with CO_2. The precipitate was very scanty, so the solution was returned to the dialyzer, without centrifugation, for 18 hours additional dialysis. The resulting precipitate, which was still very scanty, was dissolved as in the case of A, B, and C.

E. Supernatant fluid remaining from the last dialysis was concentrated in a warm air current, tested for toxicity, and for presence of globulins by the additions of increasing amounts of $(NH_4)_2SO_4$.

The result of all the toxicity tests is recorded in Table 4.

TABLE 4

Dialysis of Anaphylatoxin at 45 C., Attempted Recovery of Total Globulin

Solution	Guinea-Pig No.	Guinea-Pig Weight	C C Intra-venously	Serum Equiva-lent	Result
A (Euglobulin 18-hour dialysis)....	1	210	8.0	4.0	+3′30″
	2	195	5.0	2.5	+5′30″
	3	200	2.5	1.25	Moderate
	4	192	4.0	2.0	+5′30″
B (Euglobulin CO_2)...............	5	192	8.0	4.0	Moderate
	6	200	10.0	5.0	Moderate
C (Euglobulin 35-hour dialysis)....	7	192	6.0	3.0	Nil
	8	200	10.0	5.0	Very slight
D (Euglobulin 53-hour dialysis)....	9	190	6.0	3.0	Nil
	10	200	10.0	5.0	Very slight
E (Supernatant, albumin).........	11	200	4.0	4.0	Nil
	12	205	6.0	6.0	Moderate

It will be noticed that only in the case of the globulin from the 12-hour dialysis is any appreciable amount of toxin recovered, and in this case only 5.5 out of a possible 31 m. l. d. It is probable that the high temperature of this experiment helped to destroy much of the already unstable toxic principle. It is worthy of note that tests made on solution E showed no precipitate with 44 and 47% saturation, a faint opalescence with 50%, and a marked precipitate with 57% saturation with $(NH_4)_2SO_4$.

Of the various methods of dialysis attempted, that in which the serum is first acidified is by long odds the method of choice. In the case of ordinary dialysis for 24 hours the yields are scanty, and at best most irregular. This is probably due, in the case of dialysis at ordinary temperatures, to the long time necessary to obtain an appreciable yield of euglobulin (for euglobulin precipitation was always incomplete at the end of 24 hours' dialysis), and in the case of that at 45 C. to the effect of the comparatively high temperature on the toxic principle.

FRACTIONATION OF ANAPHYLATOXIN BY DILUTION AND ACIDIFICATION

On account of the objections to the method of dialysis just described, it was thought wise to attempt the precipitation of the euglobulin by the more rapid method of dilution and acidification

The first experiment was performed by diluting anaphylatoxic serum with 9 volumes of distilled water and immediately afterward passing CO_2 through the diluted fluid for 15-30 minutes.

A. The anaphylatoxin was made in the usual manner, and on test its m. l. d. was found to 0.25 c c. Ten c c of this serum were diluted with 90 c c of distilled water and CO_2 passed through for 30 minutes. The resulting flocculent precipitate was centrifugated out at 6,000 revolutions and iced for 2 hours. Following this, it was dissolved in 20 c c of 0.85% NaCl solution.

1. This solution (2 $\times$ vol.) was tested for toxicity.

2. Solution 1 being very opalescent, its dispersion was increased by the addition of ¼th volume of N/40 Na_2CO_3. The solution became clear and was tested for toxicity.

B. The supernatant remaining after centrifugation of the euglobulin precipitate was cooled to 0 C. Its volume was 100 c c, and to it were added 100 c c of 0 C. neutral saturated $(NH_4)_2SO_4$. The heavy precipitate separating out was filtered on a Buchner funnel, redissolved in 10 c c of distilled water, and dialyzed in a collodion sac for 18 hours. A slight precipitate showed that the CO_2 had not removed all of the euglobulin. This was removed by centrifugation, and the supernatant, consisting of pseudoglobulin, was concentrated in a warm air current to 9.5 c c and brought to isotonicity by the addition of 0.5 c c of 17% NaCl. Solution B was then tested for toxicity.

C. The filtrate (albumin) from the precipitate B (pseudoglobulin), was saturated with crystals of $(NH_4)_2SO_4$, filtered on a Buchner funnel, and the precipitate dissolved in 10 c c of distilled water, and dialyzed for 18 hours. Even this fraction showed traces of euglobulin. These were centrifugated out at the completion of dialysis, the supernatant concentrated to 9.5 c c isotonized with 0.5 c c of 17% NaCl solution and tested.

The results of this experiment are recorded in Table 5.

TABLE 5

THE FRACTIONATION OF ANAPHYLATOXIN BY DILUTION AND CO_2

Solution	Guinea-Pig		C C Intra-venously	Serum Equiva-lent	Result
	No.	Weight			
A (CO_2 euglobulin) 1.............	1	200	2.0	1.0	Very severe shock
	2	205	2.2	1.1	+4′20″
2.............	3	200	2.5	1.0	+3′
	4	195	2.1	0.8	Near-kill
	5	200	2.3	0.9	+3′40″
B (Pseudoglobulin)...............	6	215	3.5	3.5	Nil
	7	200	6.5	6.5	Nil
C (Albumin).......................	8	207	10.0	10.0	Nil

It will be seen by reference to Table 5 that all of the toxic principle recovered was found on the euglobulin precipitated by dilution and CO_2. The traces of euglobulin flocking out on dialysis of the pseudoglobulin and albumin fractions were tested for toxicity and found to produce no effect. The number of lethal doses recovered on the euglobulin A_2 may be calculated to be 10/0.8, or 12. The

original anaphylatoxin before dialysis contained 10/0.25, or 40 lethal doses. Consequently, 30% of the original toxicity was recovered on the water-insoluble globulin. No toxin could be demonstrated in this instance on the pseudoglobulin or albumin fraction, as reference to B and C (Table 5) will show. It is by no means certain, however, that some of the toxicity was not present in the water-soluble portions originally, since we have found that the toxic principle is quite unstable in the presence of large amounts of $(NH_4)_2SO_4$; this is likewise the case when high concentrations of $MgSO_4$, $MgNa_2(SO_4)_2$, and NaCl are used in fractionation.

Anaphylatoxin from this same batch was used for a test with dilution and acetic acid. The material was treated exactly as in the foregoing experiment except that 1.6 c c of N/10 acetic acid, instead of CO_2, were added to 100 c c of the dilute serum. In this case the salt alkali euglobulin was fatal to guinea-pigs in a dose of 1 c c serum equivalent. The results differ from those of the preceding experiment, however, in that some of the toxic principle could be demonstrated on the water-soluble globulin (equivalent of B, Table 5). The lethal serum-equivalent of this fraction was 3.5 c c. The total number of lethal doses recovered in this experiment was 12.8 on the euglobulin, and 2.8 on the pseudoglobulin. The total toxicity recovered was 33% of the original.

It is not to be inferred that the recoverable toxic principle is always confined to the euglobulin when anaphylatoxin is fractioned by the dilution and CO_2 method. In one instance where a sample of anaphylatoxin with a m. l. d. of 0.25 was diluted with 9 volumes of distilled water and CO_2 passed through for 30 minutes, 1 c c of euglobulin dissolved in twice the original volume of alkaline NaCl solution was fatal to a guinea-pig of 200 gm. weight. The supernatant was concentrated to original volume, made isotonic, and found to be fatal in 0.9 c c dose. Bearing in mind that the original 10 c c of anaphylatoxin contained 10/0.25 or 40 m. l. d., and that 10/1.0 were isolated on the euglobulin and 10/0.9 on the pseudoglobulin-albumin fraction, a total of 21, or more than 50% were recovered in this instance. The water soluble proteins were not in this instance fractioned with $(NH_4)_2SO_4$, a fact which strengthens the hypothesis as to the cause of loss of toxicity in the preceding experiment.

It is a curious fact that the yields of anaphylatoxin recovered after fractionation of anaphylatoxin prepared by the less efficient direct

gel method are relatively greater than when the sol-gel method is used. This has been found to be the case with both rat and guinea-pig serum anaphylatoxin, and is well illustrated by the following experiment.

Six c c of fresh rat serum were shaken up with 1.25 c c of 0 C. agar gel and the mixture incubated for 30 minutes. The agar was then removed by centrifugation and the lethal dose, on test, was found to be 0.75 c c for guinea-pigs of 200 gm. weight.

A. Four and one-half c c of this anaphylatoxin were diluted with 36 c c of distilled water and CO_2 was passed through this dilute serum for 20 minutes. The euglobulin precipitate was removed by centrifugation and dissolved in 2 volumes (9 c c) of 0.85% NaCl solution. The solution, which was opalescent, was cleared with 2 c c of N/40 Na_2CO_3, and tested for toxicity on guinea-pigs.

B. The supernatant, consisting of pseudoglobulin and albumin, was concentrated to original volume, made isotonic, and tested as in the case of A.

The results of this experiment are given in Table 6.

TABLE 6

FRACTIONATION OF DIRECT GEL RAT ANAPHYLATOXIN

Solution	Guinea-Pig		C C Intra-venously	Serum Equiva-lent	Result
	No.	Weight			
A	1	197	3.7	1.5	Light shock
	2	190	4.9	2.0	+6′30″
B	3	205	3.0	1.5	Severe shock
	4	212	4.0	2.0	+3′50″

Calculation shows that a total of approximately 75% of the original toxicity was recovered in this instance — 37.5% on the CO_2 euglobulin and 37.5% on the water-soluble proteins.

Similar experiments were made with guinea-pig serum. Here the same phenomenon held true, that is, the anaphylatoxin of comparatively low potency prepared by the direct gel method yielded far more of the toxic principle on fractionation than did more highly toxic serum prepared by the sol-gel method. The precipitation of the euglobulin by the dilution and CO_2 method gives the greatest yield of toxin in the case of guinea-pig serum. In several instances 75% of the total original toxicity were recovered on the water-insoluble globulin.

Repeated attempts were made to fractionate anaphylatoxins directly with $(NH_4)_2SO_4$, but the results were very disappointing. It is well known that single precipitations yield fractions by no means pure. Haslam has shown that at times 17 reprecipitations are required to obtain pure products. We attempted fractional precipitation of

anaphylatoxic serum with $(NH_4)_2SO_4$ and found that while a large amount of the toxic principle can be recovered on the first precipitates, purification results in a rapid disappearance of all of the toxic principle. The attempts were consequently abandoned.

Hardy and Gardiner[25] have described a method of isolating the total serum protein, freed from all but a minimal amount of other serum constituents, and with the various physiologically active principles intact. The method consists essentially in the precipitation of the serum in the cold with several volumes of absolute alcohol or acetone, and the subsequent careful extraction of the precipitate with ether. The resulting protein powder is easily soluble in distilled water, and by reason of the removal of a large amount of lipoid substance, is subject to more clear cut fractionation than the whole serum.

PRECIPITATION OF ANAPHYLATOXIC PROTEIN BY THE METHOD OF HARDY AND GARDINER

The first experiment made was a determination of the amount of anaphylatoxic principle recoverable by this method.

Rat anaphylatoxin was made by the usual sol-gel method and found to be fatal to guinea-pigs of 200 gm. weight in dose of 0.25 c c. Twenty-four c c of this serum were cooled to 0 C. and thrown into 120 c c of —8 C. absolute alcohol. The resulting heavy precipitate was at once filtered off by suction on a previously chilled Buchner funnel and washed with cold anhydrous ether Following this the precipitate was transferred to a beaker and extracted for 30 minutes with boiling ether. The resulting product, which was considerably caked, was comminuted with a spatula and transferred to a desiccator, where it was kept in vacuo over H_2SO_4 till thoroughly dry.

On the following day the powder was dissolved in original volume (24 c c) of distilled water, forming a perfectly clear, golden yellow solution. This solution, tested by intravenous injection into guinea-pigs of 200 gm. weight gave a very severe shock in 0.3 c c and was lethal in 0.35 c c.

As is the case with most principles whose activity depends to a great extent on their colloidal nature, this protein powder forms an excellent means of preserving the anaphylatoxin for great lengths of time. Acetone proved to be even more effective than alcohol, repeated trials with the former reagent giving products which retained the toxic principle quantitatively. It is of the highest importance to use cold reagents in performing this experiment. The serum should be cooled to 0 C. and the acetone to —8 to —14 C. It is advisable, moreover, to provide a jacket for the Buchner funnel in order that this may be kept at as low a temperature as possible.

[25] Proceedings Physiol. Soc., reprinted in Jour. Physiol., 1910, 40, p. 68.

Having determined that the anaphylatoxic principle is very well preserved by this method the next step was to discover whether solutions of this toxic protein would yield highly toxic fractions.

Dry rat anaphylatoxin protein equivalent to 5.5 c c of serum was dissolved in 11 c c (double volume) of distilled water. On test the m. l. d. of this protein solution was found to be 0.35 c c. This solution was diluted with 10 volumes of distilled water and CO_2 passed through it for 30 minutes. The resulting precipitate was centrifugated out at 8,000 r. p. m.

A. 1. The euglobulin precipitate was dissolved in 22 c c of 0.85% NaCl (4 $\times$ volume) and tested.

2. Solution 1 being very opalescent, the dilution was increased to 8 volume and tested.

3. A slight opalescence persisted in solution 2—it was accordingly treated with 2 c c of N/40 Na_2CO_3 and as a result immediately became water clear. This was tested as before.

B. The supernatant from A was cooled to 0 C. and was thrown into an equal volume of 0 C. neutral saturated $(NH_4)_2SO_4$ solution. The precipitate (pseudoglobulin) was centrifugated out, dissolved in 10 c c of distilled water and dialyzed in a thin collodion sac for 24 hours. A small precipitate consisting of a trace of euglobulin resulted. This was centrifugated out, dissolved in NaCl solution and tested. The supernatant was concentrated to original volume, made isotonic and tested.

C. The supernatant from the precipitate B, coming down on half saturation with $(NH_4)_2SO_4$ was concentrated in a warm air current till crystals of the salt appeared, showing full saturation. The precipitate was filtered off on a Buchner funnel, redissolved, reprecipitated, and finally redissolved in distilled water and dialyzed for 24 hours. No precipitate resulted. The dialysee was concentrated to original volume and isotonized, following which it was tested for toxicity.

The result of these tests is recorded in Table 7.

TABLE 7

THE FRACTIONATION BY CO_2 AND $(NH_4)_2SO_4$ OF HARDY PROTEIN FROM RAT ANAPHYLATOXIN

Solution	Guinea-Pig		C C Intra-venously	Serum Equiva-lent	Result
	No.	Weight			
A (Euglobulin) 1	1	175	2.8	0.7	Severe shock
	2	180	3.2	0.8	Severe shock
	3	183	3.6	0.9	+4′10″
2	4	170	6.4	0.8	+3′30″
	5	200	5.6	0.7	Very severe shock
3	6	180	6.0	0.625	Near-kill
B (Pseudoglobulin)	7	210	3.5	3.5	Nil
C (Albumin)	8	205	5.5	5.5	Nil

The result is an excellent one and surpasses by far, in regard to yield of toxin, the ones obtained by similar fractionation applied to raw serum. The original protein solution contained 5.5/0.35, or 15 m. l. d.— 5.5/0.625, or 8 m. l. d., were recovered on the euglobulin

fraction, and to this must be added at least 1 m. l. d. obtained from the trace of euglobulin coming down during the dialysis of B (pseudoglobulin), for this, when dissolved in 1 c c of NaCl solution and injected, killed a guinea-pig of 200 gm. weight. This total of 9 m. l. d. out of a possible 15 recovered gives a yield of nearly 60% on the euglobulin fraction.

The high toxic yields after fractionation were not confined to the method of dilution and CO_2, but were surprisingly good when the Hardy protein was half saturated with $(NH_4)_2SO_4$. This method, when applied to anaphylatoxic whole serum gave most inconstant results. On the other hand, when anaphylatoxic protein isolated by the Hardy method was treated in this manner and the resulting fractions dialyzed, the resulting euglobulin precipitate, when dissolved and injected, proved to possess 50% of the total original toxicity. The pseudoglobulin and albumin fractions, injected in large doses, proved to be nontoxic.

Besides the high yields of toxic principle recovered by fractionation of the anaphylatoxic Hardy protein, another result of the greatest importance should not be overlooked. That is the relatively specific manner in which the toxin is associated with the euglobulin fraction. In one case only, where the method used was the just described half saturation with $(NH_4)_2SO_4$ followed by dialysis, did we succeed in recovering any toxicity on the water-soluble globulin. In this instance out of a total of 9.2 m. l. d. recovered, 45 out of a total of 68% were demonstrated on the euglobulin. The remainder was about equally distributed between the pseudoglobulin and albumin fractions.

A point in this experiment well worthy of note is the character of the shocks and necropsy pictures in the guinea-pigs dying from the pseudoglobulin and albumin injections. In both instances the shock was of the subacute type described by one of us in a preceding paper.[26] The deaths, instead of taking place in 2-4 minutes, as is usually the case, occurred in 7 minutes 50 seconds in the case of the pseudoglobulin, and in 51 minutes in the case of the albumin. A bloody foam exuded from the nostrils during the shock, and a marked pulmonary edema was demonstrable at necropsy. This picture is characteristic of the shock produced by the injection of primarily toxic normal and immune rabbit serums, and as we shall demonstrate below, the toxin in these instances is strictly associated with the water-soluble proteins of such serums.

[26] DeKruif: Jour. Infect. Dis., 1917, 20, p. 717.

It might be well at this point to sum up the results obtained by the various methods of fractionation so far described. Dialysis of the whole serum anaphylatoxin yields at best inconstant results. The unreliability of this method appears to be due to the time consumed in the dialysis and the incompleteness of the euglobulin precipitation. The euglobulin (water-insoluble) fraction which carries the greater part of the toxicity is known to be sensitive to changes produced by too long contact with distilled water, and it is this fact that very probably is the cause of the failure to recover large amounts of toxin by this method. When the speed of dialysis flocculation of the euglobulin is increased by the addition of acid to the serum the results obtained are much better, as much as 32% of the total original toxicity being recoverable on the euglobulin fraction.

Dilution and acidification of the whole serum anaphylatoxin has the advantage of speed over even the acid method of dialysis just described. The toxin yields are quite constant, although they are not much greater in amount than those obtained by acid dialysis. In the majority of instances the greater part of the toxic principle recovered is associated with the water-insoluble globulin fraction, but in some cases a partition between the water soluble and insoluble fractions occurs. The method of preparation of the anaphylatoxin seems to affect the yield of this substance obtained on fractionation. The less efficient method of the addition of agar gel to serum giving better relative results than the sol-gel method.

When the serum proteins from anaphylatoxin are precipitated by alcohol or by acetone in the cold and extracted with dry ether, the anaphylatoxic principle can be recovered quantitatively on redissolving these proteins. The quantitative recovery is most regularly obtained when acetone is used as the precipitant. It is highly desirable to have the reagents and the apparatus used thoroughly chilled. Such protein solutions yield much higher proportions of the toxic principle on fractionation than do whole serum anaphylatoxins. What is more, the toxic principle recovered appears almost invariably and exclusively on the euglobulin.

THE ULTRA-FILTRATION OF ANAPHYLATOXIN

The methods of fractionation employed in the first part of this paper have given valuable evidence as to the location of the anaphylatoxic principle in serum. It would seem, however, in view of the delicately balanced equilibrium of the serum colloids, that the frac-

tionation procedure might introduce a too violently disturbing factor, which might be the cause of our invariable failure to recover the toxic principle quantitatively on one fraction.

If some purely physical method could be found to effect this separation of the poison from the bulk of remaining serum constituents, greater progress might be made in these studies. The first method attempted was that of ultra-filtration, devised by H. Bechhold.[27] This author is able to separate colloids of different grades of dispersion by means of membranes constructed by impregnating filter papers with gels of one kind or another. That most frequently used is an acetic acid celloidin. By varying the concentration of celloidin in the acetic acid, membranes of varying permeability are secured.

This method, after thorough trial, was discarded as unsuitable. The greatest difficulty was met with in securing any regularity in membrane permeability, and what is more, the individual membranes varied greatly in permeability at different parts of their surfaces.

Our next trials were made with the method of Schoep[28] and met with unexpectedly good results. The membrane used in this case is prepared from a solution of celloidin in alcohol-ether, the permeability of the celloidin being modified by the introduction of castor oil and glycerol. This filter is most conveniently used in the form of a dialyzing sac. The degree of permeability can be quite accurately adjusted. We will outline a description of the technic employed.

Two solutions, A and B, are prepared as follows:

Solution A

Schering's celloidin	4 gm.
Absolute alcohol	15 c c.
Dry ether	85 c c.
When dissolved add	
Castor oil	4 c c.
Glycerol	4 c c.
Absolute alcohol until clear, about	15 c c.

Solution B

Schering's celloidin	4 gm.
Absolute alcohol	50 c c.
Dry ether	50 c c.

By mixing solutions A and B in different proportions, membranes of different peremability may be prepared. The more permeable the membrane desired, the more of A is used, and vice versa. Different batches of the solutions made up at different times, do not necessarily yield membranes of exactly

[27] Ztschr. f. physik. Chem., 1907, 60, p. 237.
[28] Koll. Ztschr., 1911, 8, p. 80.

like permeability, so that the proper ratio of A to B must be determined for each batch of material made up.

Method of Making the Sacs.—The Novy-Gorsline tubes were used in preparing the sacs, the general method of preparation of which is identical with that of the ordinary celloidin dialyzing bag. A little celloidin is placed over the hole at the end of the tube and allowed to dry. The tube is then immersed in a cylinder containing the desired mixture of A and B, rotated a few times, and after gently shaking off the excess of celloidin is withdrawn from the cylinder and rotated rapidly in a horizontal position for 15-30 seconds. A second coat is now applied in the same way and if necessary a third, rotation to secure even distribution being practiced each time. The celloidin is allowed to dry on the tube until it becomes opaque and whitish. The tube is then filled with water, and after thrusting it into a large beaker of water, air pressure is applied gently to the mouth, with the result that the water within the tube passes out through the hole in the bottom and between the tube wall and the sac. The sac is thus easily disengaged from the tube.

The sac is now carefully attached to a cut-off test tube, the seal being made air-tight and tested for leakage. The apparatus is then lowered into a cylinder, and immersed in distilled water. The fluid to be filtered is introduced, the top of the cut-off test tube is closed off with a single hole rubber stopper through which the pressure line is introduced. A mercury manometer is placed in the pressure line and the filtration allowed to proceed at a pressure of not more than 100 mm. Greater pressures than this endanger the sac, the walls of which are delicate and easily ruptured.

The process of filtration usually consumes from 30 minutes to 2 hours, depending on the amount of fluid filtered and the permeability of the membrane. The volume of the fluid introduced is usually decreased from 4-6 times. The same sac can be used for more than one filtration if the precaution is taken to force a large quantity of distilled water through the bag at once after the completion of the operation.

The fluid remaining inside the bag after filtration we will designate as supernatant, that found on the outside will be called the filtrate. Since much of the electrolyte is filtered off during the operation, the supernatants were always brought to tonicity before injection, and diluted to the original serum volume. The supernatants were concentrated to a workable volume in a warm air current.

It will be apparent that this method is only of value if accurate estimation of the protein contents of the supernatant and of the filtrate is made. Only in this way can an estimation of the size of the aggregates carrying the toxic principle be formed. The refractometric method of Robertson is considered the most suitable for estimation of the protein content of serums. Unfortunately, one of these instruments was not available, so we were compelled to resort to the method of acidification, addition of salt, boiling and weighing. Filter papers dried to a constant weight were used, and in all the estimations the drying of the protein precipitates on the filters was most carefully conducted.

In the majority of instances the total protein of the supernatant and filtrate, respectively, was estimated. In cases where the euglobulin, pseudoglobulin and albumin were separately determined, the euglobulin was removed by dilution and CO_2 and redissolved. The supernatant from this precipitate was then half saturated with $(NH_4)_2SO_4$, and the thus separated pseudoglobulin and albumin fraction reprecipitated and redissolved. Finally, all three solutions were made acid to litmus with N/10 acetic, enough $(NH_4)_2SO_4$ added to make about a 10% solution, boiled for 30 minutes, and the resulting precipitates were filtered and washed with hot water until free from sulphate, dried and weighed.

The experiments with the Schoep method of ultra-filtration were conducted with both rat and guinea-pig anaphylatoxin. The size of the colloidal aggregates with which the toxic principle is associated is smaller in the case of rat serum than in that of the guinea-pig. As a result the membranes holding back the former were composed of proportionately more of solution B.

Eleven c c of rat anaphylatoxin were made by the sol-gel methol and on test the m. l. d. was found to be 0.5 c c. This serum was diluted to 55 c c with distilled water and placed in a 50A-50B sac. The sac was surrounded by a small amount of distilled water and the filtration conducted at a pressure of 80 mm. of mercury, for 1 hour and 45 minutes, until the fluid in the sac was reduced to 15 c c. Five c c of physiological salt solution were used to rinse out the sac; this was added to the supernatant, which was then brought to double the original volume by the addition of 2 c c of 17% NaCl solution. The result of the toxicity tests and protein estimation is given under A, Table 8.

The filtrate B was reduced to double volume in a warm air current. This was also tested for toxicity and a determination of protein was made.

TABLE 8

Showing Result of Toxicity Tests and Protein Estimation

Solution	Guinea-Pig		C C Intra-venously	Serum Equiva-lent	Result
	No.	Weight			
A (Supernatant)	1	200	2.0	1.0	+4′30″
	2	180	1.5	0.75	+2′55″
	3	180	1.0	0.5	Near-kill
B (Filtrate)	4	200	10.0	5.0	Nil

Of the total protein recovered, 48.3% was recovered from the supernatant A and 51.7% from the filtrate B. At the same time observation of Table 8 will show that the toxin was retained practically intact within the sac. This experiment would indicate that

the toxic principle must be part of some large colloidal aggregate that passes the ultra-filter with great difficulty, if at all, even when the porosity is great enough to pass a large portion of the serum protein.

It was thought desirable to filter anaphylatoxin repeatedly, diluting it each time after a portion of the fluid had been forced through. This experiment, the result of which proved to be interesting, was conducted as follows.

Fifteen c c of rat anaphylatoxin the m. l. d. of which was 0.35 c c, were diluted with 55 c c of distilled water and placed in a 50A-50B sac.

Filtration 1. One hundred mm. of pressure, 2 hours and 30 minutes till volume of supernatant was 30 c c. Sac then washed and 50 c c of distilled water forced through; 0.7 c c removed for test.

Filtration 2. Superanatant 1 diluted with distilled water to 50 c c, 100 mm. of pressure, 2 hours till volume was 29.7 c c. Sac washed as before. Portion removed for toxicity test.

Filtration 3. Supernatant 2 diluted to 50 c c with distilled water, 100 mm. of pressure, 1 hour and 45 minutes till volume was 26.7 c c. Tested for toxicity and protein content determined.

The filtrate from filtration 1 was concentrated to original volume and tested for toxicity and the protein content determined.

The filtrates from filtrations 2 and 3 combined, concentrated to original volume, tested for toxicity and protein content determined.

The result of the foregoing experiment is recorded in Table 9.

TABLE 9

REPEATED ULTRA-FILTRATION OF A GIVEN SAMPLE OF ANAPHYLATOXIN

Solution	Guinea-Pig		C C Intra-venously	Serum Equiva-lent	Result	Protein Content, Per Cent.
	No.	Weight				
Filtration 1						
(Supernatant)............	1	195	0.7	0.35	+3′50″	
(Filtrate).................	2	210	5.0	5.0	Nil	50
Filtration 2						
(Supernatant)............	3	190	0.7	0.35	Heavy shock	
	4	200	0.8	0.4	Very severe	
	5	210	0.9	0.45	Very severe	
	6	200	1.0	0.5	+5′40″	
Filtration 3						
(Supernatant)............	7	200	1.25	0.625	Moderate	14
	8	210	1.6	0.8	Moderate	
	9	200	2.0	1.0	+5′40″	
(Filtrate 2 + 3)..........	10	180	2.0	2.0	+4′10″	36
	11	190	1.0	1.0	Nil	
	12	175	1.5	1.5	Nil	

It will be seen from Table 9 that though half the total recovered protein was found in the first filtrate, all of the original toxicity could be recovered in the supernatant. When the supernatant was diluted a second time, filtered, and tested, there was some drop in toxicity,

the lethal doses being 0.5 instead of 0.35 c c as in the original serum and in supernatant 1. The second and third filtrates were pooled, so that the toxicity demonstrated and the quantity of protein determined must be the sum of filtrations 2 and 3. The toxicity test of these pooled filtrates shows that some of the toxic principle has passed the membrane (Guinea-pig 10, Table 9). On the other hand, the amount of toxic principle to be found here is very small, —7.5 m. l. d. This will be seen to be true, since reference to Guinea-pig 9, Table 9, shows that 13.3 m. l. d. were kept back in the third supernatant, even though this contained only 14% of the total recoverable protein.

Similar tests of multiple filtration of a given sample of anaphylatoxin confirm this finding. It is probable that repeated dilution of the toxic serum increases the dispersion of the large toxic aggregates sufficiently to allow a small amount to pass the membrane. This experiment strikingly confirms the idea of the association of the toxic principle with large colloidal complexes.

Guinea-pig anaphylatoxin was subjected to this ultra-filtration with similar results. In several instances the total toxicity was retained within the sac, while the filtrate was shown to contain at least 50% of the recoverable protein.

We have used the term "recoverable" protein in the foregoing experiments because when determinations of the protein content of unfiltered anaphylatoxin were run, values considerably in excess of the sum of the protein of the supernatant and filtrate were obtained. It was at once presumed that this discrepancy was due to adsorption of a portion of the protein in the membrane during the process of filtration. Should this be true, the results obtained would be even more striking, especially in cases where the total toxicity was recovered in the sac content after completion of filtration.

This deduction, when put to the test was found to be correct. An experiment of this kind merits detailed description because it shows the method devised for recovery of the protein held in the membrane.

Twenty c c of guinea-pig anaphylatoxin made by the sol-gel method and tested for its m. l. d. was found to be lethal in dose of 4 c c. It was diluted to 100 c c with distilled water and placed in a 5A-4B ultra-filter sac. It was filtered under 100 mm. pressure till the supernatant volume was 15 c c. One c c of 17% NaCl solution was used to make the liquid isotonic, the inside of the sac was washed with 0.85% NaCl, and this wash-water was added to the supernatant, bringing its volume back to that of the original serum (20 c c). The supernatant was now tested for toxicity and the remainder used for determination of the protein content.

The filtrate was concentrated to original volume in a warm air current, the toxicity tested and the protein content determined.

The sac used in this experiment was dried in air for 1 hour and in a desiccator for 2 hours, cut up into very small pieces and covered with alcohol and ether. This was allowed to stand overnight and in the morning, after alcohol and ether added the evening before had been decanted, was shaken up with fresh alcohol and ether and allowed to stand for 15 hours. For a control, another sac was prepared and treated in the same manner, but without being used for filtration. It completely dissolved in alcohol and ether in a few hours. The celloidin having then completely dissolved, the fluid was filtered off on a previously dried and weighed filter paper and the amount of sac protein determined by weighing.

The amount of protein in the unfiltered anaphylatoxin of the same pool was determined. The results of this experiment are recorded in Table 10.

TABLE 10

RETENTION OF SERUM PROTEIN IN ULTRA-FILTER SAC

	Amount of Protein in Gm.	Per Cent. of Protein	Toxicity (Lethal Dose)	Per Cent. of Toxicity
Supernatant	0.130	31	4.0	100
Filtrate	0.192	46	(No effect with 8 c c)	0
Sac	0.097	23		
Total	0.419	100		
Control (unfiltered anaphylatoxin)	0.413		4.0	

While the toxicity of the serum is completely recovered in the supernatant, only 31% of the total protein is to be found in this portion. The amount of the protein adsorbed in the membrane has varied in our experiments from 18-25% in the case of guinea-pig anaphylatoxin. The protein of rat anaphylatoxin seems to be retained in less amount, this varying from 7-20%. It will be apparent that the results given previous to this experiment would have been more striking, since the addition of the sac protein to that not found associated with the toxic principle would markedly increase the amount of toxin per unit quantity of protein recovered.

Of numerous experiments made with ultra-filtration the best result obtained has been the filtering off of 70% of the total protein with a total retention of toxicity within the sac. A fact worthy of note is that tests have shown the filtrates in cases where the toxicity has failed to pass the membrane to be free of water-insoluble globulin, that is, no precipitate was obtained when the filtrate was diluted and CO_2 bubbled through it for 30 minutes. On the other hand, the water-soluble (pseudo) globulin passes the membrane almost, though not quite, as freely as does the albumin. It would appear from this, then,

that while the euglobulin is not necessarily chemically different from the pseudoglobulin, its physical aggregates may be larger, possibly because of its combination with serum lipoid as suggested by Chick. It is unfortunate that we did not test the ability of normal serum euglobulin to pass these membranes. While the result might not be different, it is just possible that an actual aggregation may be a part of the process of anaphylatoxin production. Bordet,[29] indeed, observed that a heavy precipitate occurs when agar is added to unheated normal serum.

We have extracted many samples of the agar sediment centrifuged out after anaphylatoxin production and have not only succeeded in recovering considerable of the toxic principle, but by fractionation with $(NH_4)_2SO_4$ and dialysis have found the toxin to be associated with the euglobulin. It is possible then, that the whole explanation of the toxin production may lie in an aggregation of the globulin portion of serum under the influence of the colloid introduced. Plausibility is lent to this view by the peculiar phenomenon of distilled water toxification of normal and immune rat serums first observed by one of us in collaboration with Professor Novy.[30] It is well known that simple dilution produces spontaneous precipitation of the water-insoluble serum globulin. The temptation to correlate these two phenomena with those described in the preceding paragraphs is great, especially when one takes into account certain observations made in the course of this work.

We present the following observations tentatively because the phenomena observed have occurred irregularly and have so far been difficult to duplicate. This is perhaps due to the fact that we have not had time to determine all the factors which must be kept constant to bring about invariably the results we have observed a few times.

In an early paragraph it will be recalled that we made control injections of the euglobulin from normal rat serum and were able to inject 10 c c serum equivalent of this protein without any harmful effect resulting. Since the toxic principle of anaphylatoxin is found to be associated closely with the euglobulin fraction, we attempted to make rat serum toxic by the agar sol-gel method after the euglobulin had been removed by dilution and acidification or by dialysis. It was found that the simple removal of this water insoluble globulin, which is a rather small constituent of the total rat serum protein, made a

[29] Compt. rend. Soc. de biol., 1913, 74, p. 1213.
[30] Novy and DeKruif: Jour. Infect. Dis., 1917, 20, p. 776.

profound change in the toxification reaction. The serum which before had been toxifiable in dose of 0.25 c c now could be made toxic only in very large doses (5-7 c c). In fact, this toxicity can easily have been a primary one.

Rat serum, then, loses its ability to become anaphylatoxic after removal of the water-insoluble globulin. On the other hand, when the water-insoluble globulin, freshly removed from serum, was mixed with agar and treated by the sol-gel method it apparently changed from a harmless state to one typically anaphylatoxic. It would be well to present the experiment in detail.

Nineteen c c of fresh normal rat serum were cooled to 0 C. and half saturated with cold neutral $(NH_4)_2SO_4$. The precipitate was centrifugated off and redissolved in 15 c c of distilled water and dialyzed for 24 hours. The heavy euglobulin precipitate was taken up in 19 c c (original volume) of 0.85% NaCl solution. The resulting solution was very opalescent and was therefore diluted to double volume by the addition of 19 c c more of normal salt. Twelve c c of this solution were set aside for control and the remainder treated as follows:

Twenty-three c c of euglobulin solution were warmed to 38 C. and mixed with 2 c c of 0.5% agar sol. The mixture was shaken, iced for 30 minutes, following which it was placed in the incubator for 30 minutes at 38 C. A flocculent precipitate similar to that forming when whole serum is toxified was visible at the end of this time. The agar was centrifugated out at 3,000 r. p. m. and the supernatant was tested for toxicity by intravenous injection into guinea-pigs of 200 gm. weight.

The result of this experiment is given in Table 11.

TABLE 11

THE TOXIFICATION OF RAT SERUM EUGLOBULIN BY AGAR

Solution	Guinea-Pig		C C Intra-venously	Serum Equiva-lent	Result
	No.	Weight			
Control (untreated globulin).......	1	180	11.0	5.5	Nil
Euglobulin + agar..................	2	175	7.0	3.5	+2′50″
	3	180	4.0	2.0	Moderate
	4	185	6.0	3.0	Severe

The picture of the shock and the findings at necropsy were perfectly typical of that produced by whole serum anaphylatoxin. Several attempts to duplicate this experiment resulted in failure, while several others for no explainable reason succeeded as well as the one described above. On the whole, it was found better to remove the euglobulin from fresh rat serum by dilution and CO_2 than by the method just described, for the reason that this product will undergo deleterious changes by long contact with distilled water.

These observations are of great interest because they immensely simplify the question of the mechanism of anaphylatoxin production. Instead of working with such an infinitely complex mixture of colloids as the whole serum, one is now able to study the euglobulin alone. This is far simpler than the former method, even taking into consideration the fact that the product used was not pure euglobulin.

This experiment awaits elaboration and careful control and we hope at some future time to concern ourselves exclusively with it. In the meanwhile the findings presented here support strongly the tentative hypothesis that serum toxification by foreign colloids is intimately related to some change, physical or chemical, in the water-insoluble globulin of the serum.

THE FRACTIONATION OF PRIMARILY TOXIC NORMAL AND IMMUNE SERA

This subject will only be touched on in a few words and is to be taken as a preliminary report of an investigation which will be elaborated in a later paper. It is well known that the normal serums of numerous species of animals are toxic for guinea-pigs. In the case of normal rat, rabbit, sheep and human serum we have invariably failed to find any of the primarily toxic principle associated with the water-insoluble globulin. On the contrary, in the case of sheep and rabbit serum more than 50% of this toxicity can be isolated on the pseudoglobulin fraction. The methods used in this investigation are entirely similar to those fractionations described in the first part of this paper.

It is a curious though well known fact that the serum of rabbits immunized with sheep red blood cells becomes extremely toxic for guinea-pigs. It was our good fortune, during the immunization of some rabbits with these cells, to obtain a sample of serum from a rabbit which had become cachetic under the injections. It had previously been noted by Jonesco Mihaiesti[31] that serum of rabbits becoming cachectic under immunization is extremely toxic. The sample obtained by us confirmed this statement, since the serum was fatal to guinea-pigs on intravenous injection in dose of 0.05 c c. This toxicity decreased to 0.1 c c after the serum had been kept for a few days. A sample having this toxicity was precipitated with cold alcohol and extracted with ether by the method of Hardy and Gardiner already

[31] Compt. rend. Soc. de biol., 1913, 74, p. 1414.

described. The toxicity was recovered quantitatively when the resulting protein powder was redissolved.

Upon half saturation of this toxic protein solution with $(NH_4)_2SO_4$ and dialysis of the redissolved total globulin precipitate, the toxicity was found to be concentrated almost entirely on the pseudoglobulin fraction —0.1 c c of the protein solution was fatal to guinea-pigs, while 0.2 c c serum equivalent of the pseudoglobulin fraction produced the same effect. While the water-soluble globulin thus carried at least 50% of the original toxicity the euglobulin was found to possess only 3% and the albumin fraction none at all. This is in striking contrast to the results obtained with rat and guinea-pig anaphylatoxin, in which cases, as we have seen, the poison is almost entirely concentrated on the euglobulin fraction.

SUMMARY AND CONCLUSIONS

The toxic principle of rat and guinea-pig anaphylatoxins can be recovered on the globulin fractions of these serums.

Various methods of isolating these fractions have been studied. In the case of dialysis, very little of the toxic principle is recovered unless the speed and efficiency of this procedure is increased by the preliminary neutralization of the serum. Better results are obtained when the water-insoluble globulin is removed by dilution and CO_2 or dilution and acetic acid. In all of these cases the toxic principle appears on the water-insoluble rather than on the water-soluble fraction of the globulin, although in some instances partition between these fractions is found to occur. This seems to be due to the fact that the poison associated with the water-soluble fraction is much more unstable in the presence of high concentrations of $(NH_4)_2SO_4$ and other salts than is that associated with the water-insoluble fraction.

When anaphylatoxic serums are treated by the method of Hardy and Gardiner for the removal and purification of serum proteins, the toxic principle is quantitatively recoverable on the serum proteins. Such protein solutions, when fractionated, yield far more constant, more specific, and better quantitative results than those obtained by fractionating the whole serum. The toxic principle recovered from the fractionation of these protein solutions is found to be almost invariably and exclusively concentrated on the euglobulin fraction.

Anaphylatoxic serums have been further studied by the method of ultra-filtration. By the use of Schoep's methods most instructive

results have been obtained. The toxic principle has been shown to exist in extremely large colloidal aggregates which are retained even after as much as 70% of the total serum protein has passed the membrane. Quantitative determinations would indicate that very little or no water-insoluble globulin passes the filter, even when from 50% of the total protein has been recovered in the filtrate.

The foregoing results are important in that they furnish an excellent and simple means of concentrating the toxic principle, furnish material for a tentative hypothesis of the nature of the toxin, and effectively exclude the notion that protein degradation products are concerned in this toxicity. (The toxic principle of Witte peptone passes easily membranes which hold back all of the anaphylatoxin.)

It appears that euglobulin solutions freshly prepared from rat serum are toxifiable by the addition of agar. The results are inconstant, but have been reduplicated sufficiently to warrant their mention in this place.

A preliminary report of the fractionation of primarily toxic normal and immune serums has been made. Such toxins are not found on the euglobulin fraction as in the case of anaphylatoxin, but are usually recovered in the greatest concentration on the water-soluble globulin.